B_{12} Vitamins (Cobalamins)

1. VITAMINS B_{12c} AND B_{12d}

By E. LESTER SMITH, K. H. FANTES, S. BALL, J. G. WALLER, W. B. EMERY, W. K. ANSLOW and A. D. WALKER
Glaxo Laboratories Limited, Greenford, Middlesex, and Barnard Castle, Co. Durham

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During 1949 we isolated three red crystalline anti-pernicious anaemia factors from *Streptomyces griseus* fermentation liquors. The first of these was vitamin B_{12} itself, originally isolated by Rickes, Brink, Koniuszy, Wood & Folkers (1948) and a little later in these laboratories (Lester Smith & Parker, 1948). The next was vitamin B_{12b}, the second factor we obtained from liver; it was later crystallized by Pierce, Page, Stokstad & Jukes (1949). The third was vitamin B_{12c}, which has been referred to under its temporary designation 'vitamin B_{12x}' at scientific meetings (Lester Smith, Ungley, Mollin & Dacie, 1950; Buchanan, Johnson, Mills & Todd, 1950*a*, *b*; Anslow *et al.* 1950; Lester Smith *et al.* 1951). These were all obtained by methods of isolation unlikely to alter the factors chemically. In addition, however, we have obtained a number of other closely related factors by mild chemical treatments of these B_{12} vitamins (Lester Smith, Ball & Ireland, 1952).

On hydrolysis with 5N-HCl followed by shaking with *n*-octanol, vitamins B_{12} and B_{12c} gave red esters with identical absorption spectra, indicating that a major portion of the molecule is common to both (Fantes & Ireland, 1950).

Nature of vitamin B_{12c}

The yield of the new factor, vitamin B_{12c}, was very small (e.g. 3 mg. from 2500 l. of fermentation liquor), but it was enhanced (e.g. to 60 mg. from 2500 l.) at the expense of the vitamin B_{12b} by a chemical step introduced to simplify the purification procedures. This was treatment with nitrous acid, which, by deaminating amino-acids and peptides, facilitates their removal from the crude concentrate. Nitrous acid appears to have no effect on vitamin B_{12} itself, but it readily converts vitamin B_{12b} into vitamin B_{12c}. Since the former is basic, while vitamin B_{12c} (like vitamin B_{12} itself) is not, the reaction was at first interpreted as a straightforward deamination. This explanation became dubious when we found that the product, vitamin B_{12c}, could readily be converted into a basic factor, which we called vitamin B_{12d} (Lester Smith *et al.* 1951); it arose not only on catalytic hydrogenation but also from some mild hydrolytic procedures. Experiments along these lines, however, showed some puzzling features. Thus a solution of vitamin B_{12c} at pH 4·5 was not appreciably altered on autoclaving in a sealed ampoule, yet the same solution on autoclaving in a flask plugged with cotton wool showed partial conversion to the basic factor; the two could be separated by partition chromatography on kieselguhr or paper, and were identified by differences in their absorption spectra, partition coefficients and chromatographic behaviour (see below, p. 393). Again, merely concentrating a similar solution under reduced pressure at a much lower temperature caused complete conversion to the basic factor. Standing in the cold in alkaline solution caused little change, yet ionophoresis in agar jelly at pH 9 (Gordon, Keil & Šebesta, 1949) brought about complete conversion. Evidently an equilibrium was set up in acid or alkaline solutions, and complete conversion depended upon the removal of one component from the system; moreover, this component was clearly volatile to some degree. Accordingly, acidified solutions of vitamin B_{12c} were distilled into very dilute alkali and the distillates were tested. They gave a blue colour with acidified starch iodide solution, and more specific colour reactions for nitrite with *m*-phenylenediamine or with sulphanilic acid and α-naphthylamine. A roughly quantitative colorimetric assay with these latter reagents indicated 0·65 mole of nitrite per molecule. Part of the nitrous acid would have been lost by oxidation during the distillation. We therefore concluded that the molecule of vitamin B_{12c} incorporates one nitrite group, probably linked directly to the cobalt atom.

Further confirmation of this relationship is given by the observation that rapid conversion of vitamin B_{12c} to the basic factor occurs in the cold on treating acidified solutions with either sulphamic acid or urea, reagents that rapidly destroy nitrous acid. After 5 min., the reaction went to completion with sulphamic acid, but only about half-way with urea.

A compound, presumably identical with vitamin B_{12c}, has recently been described briefly by Kaczka, Wolf, Kuehl & Folkers (1951) under the name nitrocobalamin. The nomenclature of these compounds is discussed by Lester Smith, Ball & Ireland (1952).

It is somewhat surprising to find such a compound as vitamin B_{12c} arising from a fermentation (for, as mentioned above, it is present in concentrates that have not been treated with nitrous acid). However, it is not necessarily synthesized directly by the mould; a trace of nitrite in the fermentation liquor (possibly arising from oxides of nitrogen in the air flow) could convert part of the vitamin B_{12b} present into vitamin B_{12c}.

Nature of 'vitamin B_{12d}'

The work described above was done before anything was known of the constitution of vitamin B_{12}, and we believed that nitrous acid caused some chemical change in vitamin B_{12b} besides being added on reversibly. In that event, the denitrosation product, which we called vitamin B_{12d}, would differ from vitamin B_{12b}. The absorption spectra were identical, including the shift at alkaline pH values already recorded for vitamin B_{12b} (Brockman, Pierce, Stokstad, Broquist & Jukes, 1950). Such spectroscopic evidence would normally be accepted as evidence of identity; in this range of molecular dimensions, however, we considered that a small difference in structure would not necessarily alter the absorption spectrum appreciably. The need for such caution has since been emphasized by the observation of Buhs, Newstead & Trenner (1951) that the absorption spectrum of vitamin B_{12b} is not altered on conversion[1] to thiocyanatocobalamin. Partition chromatography would probably be the most sensitive technique to reveal such small differences, and it did appear to separate vitamins B_{12b} and B_{12d}. Thus kieselguhr partition chromatograms of concentrates (run with wet *n*-butanol, see Experimental section) sometimes showed an additional slow band, i.e. four bands in all, due respectively to vitamins B_{12c}, B_{12}, B_{12b} and (we presumed) B_{12d}. The non-identity of vitamins B_{12b} and B_{12d} was apparently confirmed by partition chromatography of the crystalline preparations (1–2 mg.) on 1 cm. diameter columns of kieselguhr carrying half its weight of pH 4·5 buffer (2% potassium dihydrogen phosphate) with wet *n*-butanol as mobile phase. Vitamin B_{12b} gave a slow-moving band ($R = 0{\cdot}1$ approx.) with only a trace of a slightly faster one: 'vitamin B_{12d}' gave a band that appeared to correspond with the latter ($R = 0{\cdot}2$ approx.), accompanied by a trace of a slower band, while a mixture gave two nearly equal bands. It now seems that these results were fortuitous. A recent reinvestigation (Lester Smith, 1952*a*) has shown that chromatograms of these three patterns, as well as columns showing a single band, can all be produced at will from the same specimen of vitamin B_{12b} by making slight changes in the operating procedure.

When the mobile phase was altered to 15% phenol in *n*-butanol (saturated with water) to secure a higher R value (0·25), a single band was seen consistently. Under these conditions mixtures of vitamins B_{12b} and 'B_{12d}' could not be separated. Their identity was confirmed by tracer techniques, using 'vitamin B_{12d}' mixed with vitamin B_{12b} containing radioactive cobalt (Lester Smith, Hockenhull & Quilter, 1952). On butanol columns two bands appeared; both, however, were radioactive, and the specific activities of the materials extracted from the two bands were the same. Thus the apparent separation of two entities was spurious. On the phenol-butanol column, however, only one band appeared: it was dissected into upper, middle and lower segments, which were separately eluted, yielding products of identical specific activities (within experimental error). This is strong evidence of identity, since a radioactive and a non-radioactive substance having partition coefficients differing by only a few per cent (i.e. not enough to cause separation into two bands) would still show a difference in specific activity between the top and bottom of the band (Udenfriend, 1950).

A similar experiment was carried out with a mixture of vitamin B_{12b} prepared from a fermentation with *Streptomyces aureofaciens* (kindly supplied by Dr T. H. Jukes of Lederle Laboratories) and radioactive 'vitamin B_{12d}'. The partition chromatogram showed a single red band ($R = 0{\cdot}25$); the upper and lower sections of this band gave materials of identical specific activity. Thus the two tracer experiments confirm the identity of 'vitamin B_{12d}' with vitamin B_{12b}, whether of natural origin or made from vitamin B_{12} by photolysis.

Basicity of vitamin B_{12b}

The basicity of vitamin B_{12b} was first revealed by its more complete extraction from alkaline than from neutral or acidic solutions by organic solvents, such as benzyl alcohol or phenol-butanol mixtures (Lester Smith, Ball & Ireland, 1952). Samples crystallized from neutral solutions behaved like salts of bases on electrometric titration in aqueous solution. The free base was prepared by passing an aqueous solution through a column of strongly basic anion-exchange resin (Experimental resin FF/X4 supplied by Permutit Ltd.), followed by crystallization from aqueous acetone. An aqueous solution (0·2%) of vitamin B_{12b} so treated had a pH value around 9 and consumed one equivalent of acid on electrometric titration (Fig. 1); the pK_a value was 7·5 (see also Kaczka, Wolf, Keuhl & Folkers, 1950).

Absorption spectra

The infrared absorption spectra of vitamins B_{12c} and B_{12b} are given in Fig. 2; the measurements were made by Dr N. Sheppard at the University of Cambridge. The main ultraviolet absorption band of vitamin B_{12c} in neutral aqueous solution occurs at

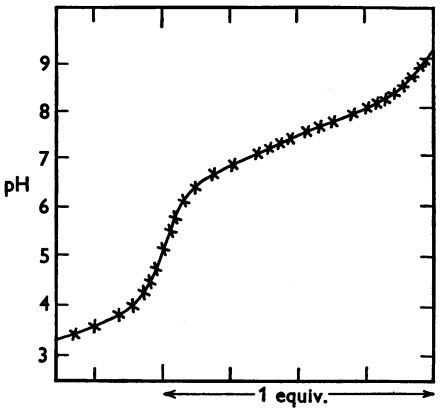

Fig. 1. Electrometric titration of vitamin B_{12b} (2·14 mg. vitamin B_{12b} base in 1·0 ml.).

352 mμ., that of vitamin B_{12b} at 351 mμ.; the former is also distinguishable by its greater breadth, the half-band width being about 42 mμ. compared with 30 mμ. for vitamin B_{12b}. Since this behaviour could be exhibited by a mixture of factors with slightly different absorption maxima, vitamin B_{12c} was subjected to rigorous purification. Recrystallization ten times from aqueous acetone did not change its absorption spectrum, and partition chromatography on kieselguhr with *n*-butanol revealed no sign of heterogeneity.

A number of physical measurements have been undertaken on vitamins B_{12b} and B_{12c}. The measurements include polarography, optical rotation, electrometric titration and electrical conductivity, and they were carried out on a micro scale in the same way as those reported for vitamin B_{12} (Fantes, Page, Parker & Lester Smith, 1949).

Polarography of vitamins B_{12b} and B_{12c}

The polarographic behaviour of vitamins B_{12b} and B_{12c} (recrystallized ten times) are summarized in Tables 1 and 2.

In neutral solution, both vitamins B_{12b} and B_{12c} formed characteristic waves with pronounced maxima; the half-wave potential was approximately $-1{\cdot}50$ V. The maximum was only partly suppressed by the addition of 0·01 % of gelatin. In alkaline and acid solution, the wave was much lower and the maximum disappeared. This behaviour is similar to that observed for vitamin B_{12} itself and is associated with catalytic waves rather than with normal reduction steps. It provides additional evidence that the cobalt atom is bound in a complex and is not ionizable. This work was completed before the appearance of papers on the polarographic behaviour of vitamin B_{12} by Diehl, Sealock & Morrison (1950) and Diehl, Morrison & Sealock (1951). These authors, by working with a higher concentration of vitamin B_{12} (9×10^{-4} M as opposed to $0{\cdot}6 \times 10^{-4}$ M), were able to identify a reduction step at $-1{\cdot}12$ V in addition to the catalytic wave.

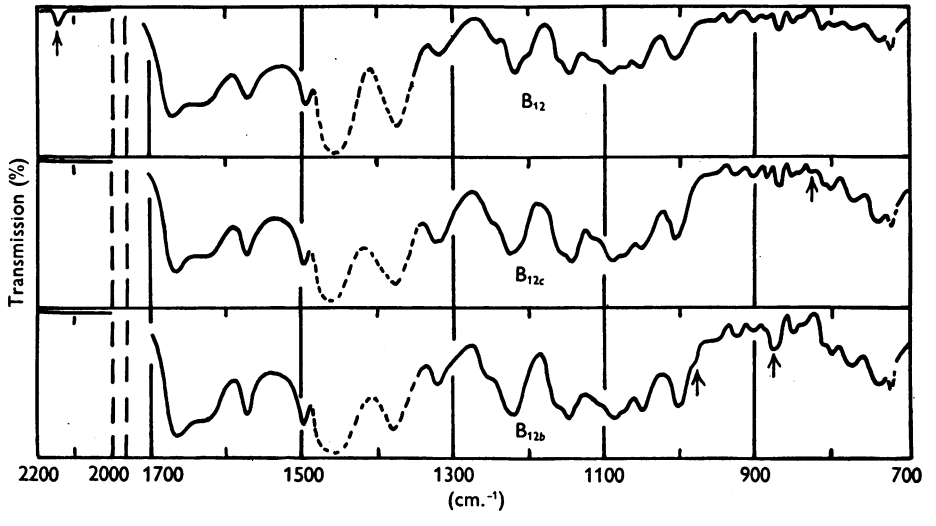

Fig. 2. Infrared absorption spectra of vitamins B_{12}, B_{12b} and B_{12c}. - - - - -, Nujol absorptions.

Table 1. *Polarographic behaviour of vitamin B_{12b}*

(The dropping mercury electrode at a pressure of 55·5 cm. of mercury and on open circuit in 0·1 N-KCl at 25° had a drop time of 3·13 sec.; the weight of the mercury dropping was 1·82 mg./sec. The half-wave potentials were measured against a saturated calomel electrode.)

Concn. of anhydrous vitamin B_{12b} (%)	pH value of solution	Half-wave potential (V.)	Diffusion current (μa.)	
			Maximum	Minimum
0·0060	7·4	−1·50	9·6	1·3
0·0058	10·8	−1·55	—	0·38

Table 2. *Polarographic behaviour of vitamin B_{12c}*

(Conditions as in Table 1.)

Concn. of anhydrous vitamin B_{12c} (%)	pH value of solution	Gelatin (%)	Half-wave potential (V.)	Diffusion current (μa.)	
				Maximum	Minimum
0·0093	3·3	0	−0·78	—	0·38
0·0090	3·3	0·01	−0·93	—	0·28
0·0096	7·2	0	−1·50	17·3	4·0
0·0090	7·2	0·01	−1·52	3·4	3·2
0·0090	9·6	0	−1·55	0·64	0·47
0·0085	9·6	0·01	−1·55	0·35	0·31

Electrical conductivity of vitamin B_{12c}

At a concentration of about $5·9 \times 10^{-4}$ M, the specific conductivity of vitamin B_{12c} at 25° was 35 gemmhos. This value corresponds to an equivalent conductivity of about 59; the latter compares with a figure of 53 obtained for vitamin B_{12}. Vitamin B_{12c} is, therefore, weakly ionized in aqueous solution.

Magnetic susceptibility

A sample of vitamin B_{12c} was kindly examined by Dr Corona Trew, who found it to be diamagnetic, like vitamins B_{12} and B_{12b} (Diehl, Haar & Sealock, 1950; Wallmann, Cunningham & Calvin, 1951).

Optical rotation of vitamins B_{12b} and B_{12c}

The optical rotations have been measured in a 0·5 dm. micro-polarimeter tube having a capacity of 0·4 ml. The polarimeter was fitted with a direct-vision spectroscopic attachment, and an experimental compact source type cadmium lamp (kindly lent by Mr B. S. Cooper of the Research Laboratories of the General Electric Co.) was used as light source. The specific rotations for light of wavelength 643·8 mμ. of solutions at 20° containing about 0·5% of vitamin B_{12} (for comparison), vitamins B_{12b} and B_{12c} are listed in Table 3. It is difficult to obtain accurate results on these solutions, especially in a micro-tube, owing to their intense colour, which also prohibits the use of higher concentrations. Therefore a considerable number of individual readings were made on each and the means with their fiducial limits (at $P = 0·05$) were calculated.

We were surprised to find that vitamin B_{12c} rotates in the opposite direction from vitamins B_{12} and B_{12b}. Brink *et al.* (1949) reported that vitamin B_{12} has a specific rotation of $-59° \pm 9°$ at 23° for light of wavelength 656·3 mμ.

Table 3. *Specific rotation of vitamins B_{12}, B_{12b} and B_{12c} in 0·5% aqueous solutions*

Vitamin	Mean specific rotations at 643·8 mμ. (°)	Fiducial range ($P = 0·05$) (°)
B_{12}	−110	−134 to −87
B_{12b}	−19·5	−28·0 to −11·0
B_{12c}	+50	+41·5 to +59·0

Clinical activity

Vitamins B_{12c} and B_{12b} are just as effective as vitamin B_{12} itself against pernicious anaemia (Lester Smith *et al.* 1950; Ungley, 1951; Chalmers, 1951; Reid, 1951).

EXPERIMENTAL

Isolation of vitamin B_{12c}

The submerged fermentations of *Streptomyces griseus* were carried out on soybean medium (Kirkpatrick, 1947; Dulaney, Ruger & Hlavac, 1948). The fermentation liquor usually contained 0·5–1 μg./ml. of vitamin B_{12} activity assessed by microbiological assay with *Lactobacillus leichmannii* (Emery, Lees & Tootill, 1951). The broth was acidified with H_3PO_4 to pH 2·0–2·5 and filtered with the aid of kieselguhr (Hyflo Super-Cel) on a rotary filter. It was similarly refiltered after adjustment with NaOH to pH 7·5. The filtrate was heated at 100° for 15 min. to release B_{12} vitamins from bound forms.

The following purification procedure is typical; sometimes steps were omitted or modified slightly, or their order was changed. A portion of the liquor (4500 l.) was stirred for 0·5 hr. with charcoal (45 kg., Sutcliffe Speakman no. 5 as purchased). The charcoal was removed by means of a rotary filter precoated with kieselguhr and was washed with 6% aqueous phenol (2250 l.) to remove some adsorbed impurities. This washed adsorbate was then stirred with a further 4500 l. of fermentation liquor and was again washed with 6% phenol. These procedures were repeated with a third portion of liquor and of 6% phenol. The charcoal adsorbate was stirred with 90% aqueous phenol (170 kg.) for 0·5 hr., the mixture filtered and the charcoal re-extracted with a second portion of 90% phenol. The combined phenol eluates were stirred with ether (1400 l.) and water (120 l.). After allowing to settle overnight, the aqueous layer was run off and two further extractions made, each with 60 l. of water.

The combined aqueous extracts were adjusted to pH 4 with HCl and filtered. The filtrate was concentrated under reduced pressure to 15 l. This concentrate usually contained about 200 μg. of vitamin B_{12} activity and about 100 mg. of total solids per ml.

When treatment with nitrous acid was included it was carried out at this stage by adding $NaNO_2$ (0·75 kg.) and 5N-HCl to pH 3·8–4·2. After standing for 0·5 hr. and readjustment of pH if necessary, the solution was filtered. To remove some impurities the solution was extracted with 3×1 vol. of *n*-butanol, maintaining the volume and pH of the aqueous phase by addition of water and HCl as necessary. The aqueous layer was adjusted to pH 7·3–7·7 with 5N-NaOH, filtered and extracted with 3×1 vol. of phenol-butanol (1:2 by volume). The combined solvent extracts were washed with 2×5 l. portions of water.

The phenol-butanol extract was stirred with ether (180 l.) and water (15 l.). After separating the aqueous layer, two further extractions were made, each with 7·5 l. of water. The combined aqueous extracts were re-extracted with ether and concentrated under reduced pressure to one-third the original volume.

When treatment with nitrous acid was included, it was repeated at this stage by adding $NaNO_2$ (5 g./l. of concentrate) and heating to 70–80° for 5 min., adding 5N-HCl as necessary to maintain pH 3·8–4·2.

Some impurities were removed by adding $(NH_4)_2SO_4$ (200 g./l.) and filtering through a kieselguhr bed. The filtrate was mixed with kieselguhr (20 g./l.) and additional $(NH_4)_2SO_4$ (300 g./l.), and filtered after standing for a short time. The precipitate was extracted with ethanol (1 l.) and the extraction repeated until no more colour was removed. The bulked extracts were stirred with kieselguhr (80 g.) while adding ether (3 vol.), to precipitate the active material. The precipitate was filtered off and extracted with small portions of water until the extracts were colourless.

At this stage the total solids was determined. Charcoal (Sutcliffe Speakman no. 5) and kieselguhr, each equal to four times the weight of solids in the concentrate, were slurried with water and made into a bed about 1 cm. deep on a Büchner funnel. The concentrate was filtered slowly through this bed, followed by 10% aqueous *n*-propanol at the rate of 25 l./kg. of charcoal to remove impurities. The adsorbate was then eluted with 50% aqueous propanol at the rate of 15 l./kg. of charcoal. The propanol eluate was concentrated under reduced pressure to remove the propanol and reduce the volume to about 250 ml.

Alumina (200 g., Spence Grade 0) was well washed with very dilute HCl at pH 3·8 and then with water. A short chromatogram column was prepared with this alumina and the concentrate was percolated through the column, followed by distilled water until the effluent was pale pink in colour. Brown impurities were retained on the alumina. This effluent plus washings was adjusted to pH 4 and stirred with granular charcoal (Sutcliffe Speakman no. 207 B), adding the charcoal until the supernatant was colourless. The adsorbate was well washed with water, then eluted with 50% aqueous *n*-propanol until no more red colour was extracted. The eluate was concentrated to a syrup and dissolved in *n*-butanol, adding water if necessary so that the solution was just saturated with water.

A kieselguhr partition chromatogram was prepared as follows: kieselguhr (Hyflo Super-Cel) was washed with dilute HCl, then with water and dried at 150°. The kieselguhr was stirred with about six times its weight of water-saturated *n*-butanol, while half its weight of water was added slowly with vigorous stirring. The slurry was poured into a chromatogram tube and the kieselguhr column compacted by applying gentle air pressure to the top of the column and running through additional water-saturated butanol if necessary. Columns varying from 1 to 6 in. in diameter were used for various preparations. We found it desirable to allow about 1 kg. of kieselguhr for each 0·5 g. of vitamin B_{12} activity, usually associated with about 5 g. of solids. The concentrate in wet butanol was then added and the chromatogram was developed with water-saturated butanol.

Three red bands appeared on the column (sometimes four), due to vitamin B_{12c} (*R* value about 0·4), vitamin B_{12} itself (*R* value about 0·27) and vitamin B_{12b} (*R* value about 0·05). The *R* values differed somewhat from one run to another, probably owing to variation in the tightness of packing of the moist kieselguhr on the column, but the relative *R* values did not change significantly. The faster zones were usually developed right through the column. The various red fractions generally crystallized fairly readily after concentrating, precipitating with excess of acetone, redissolving in water and adding enough acetone to give slight turbidity. If necessary they were first purified further by adsorption on charcoal.

The charcoal eluate usually contained about 35% of the vitamin B_{12} activity of the fermentation liquor. When the HNO_2 treatments were included, the yield after chromatography on alumina had fallen to about 11%. The yield of crystalline material was about 6%, of which 4 or 5% was vitamin B_{12c} and 1 or 2% vitamin B_{12} itself.

Paper chromatography

Partition chromatography on paper (Whatman no. 4) was used to distinguish vitamins B_{12b} and B_{12c}. The usual descending method of development was used with water-saturated *sec.*-butanol. Sufficient material was used (0·1 mg. or more) to give clearly visible spots. The R_F values were about 0·25 for vitamin B_{12c} and 0·05 for vitamin B_{12b} (the R_F for vitamin B_{12} is 0·2 approximately). The chromatograms were usually developed overnight when the solvent front ran off the bottom of the paper. Control spots of the pure substances were used for identification.

Preparation of radioactive vitamin B_{12b}

Radioactive vitamin B_{12} (0·1 mg. containing 1·55 μc. of ^{60}Co) (Lester Smith, Hockenhull & Quilter, 1952) was added to ordinary vitamin B_{12} (13·4 mg.) dissolved in water (50 ml.). The solution was acidified to about pH 4 and illuminated with two 500 W. lamps for several hours while a stream of N_2 was bubbled through the solution. The apparatus described by Boxer & Rickards (1951) was used and the treatment was continued until HCN could no longer be detected in the effluent gas.

To remove acid the solution was run through a column of FF/X4 resin (5 g.) previously washed with a NaOH solution followed by water. The filtrate and washings were concentrated under reduced pressure to about 0·5 ml., then excess acetone was added to induce crystallization. The product was recrystallized from aqueous acetone yielding vitamin B_{12b} with a specific activity of 0·115 μc./mg.

Preparation of radioactive 'vitamin B_{12d}'

Vitamin B_{12} containing a little [^{60}Co]vitamin B_{12} was converted into vitamin B_{12c} by treatment with sulphite followed by nitrite (Lester Smith, 1952*b*). The radioactive vitamin B_{12c} (5 mg.) was dissolved in water (1 ml.) and treated with sulphamic acid (2·5 mg.) during 1 hr. at room temperature. The solution was extracted with 1 ml. of phenol and the extract washed with two portions of 0·1 N-NaOH (2 ml.), then with water, to prepare the free base. The phenolic solution was treated with acetone (0·5 ml.) and ether (10 ml.) and centrifuged. The red precipitate was dissolved in a few drops of water and crystallized by adding excess acetone. The resulting 'vitamin B_{12d}' had a specific activity of 1·4 μc./mg.

Partition chromatography of radioactive vitamin B_{12b} and 'vitamin B_{12d}'

The solvent was 15% (w/v) phenol in *n*-butanol just saturated with water. The solvent was shaken with one-tenth its volume of 2% phosphate buffer at pH 7. Acid-washed kieselguhr (Hyflo Super-Cel, 60 g.) was suspended in the solvent mixture (360 ml.) and 30 ml. of the aqueous layer was added with vigorous stirring. Part of the slurry was poured into an 18 mm. diameter tube to form a column about 25 cm. in height after compacting by slight air pressure.

1 mg. each of radioactive vitamin B_{12b} and ordinary 'vitamin B_{12d}' were dissolved in about 2 ml. of the solvent and added to the column. The chromatogram was developed until the red band nearly reached the bottom. The column was then sucked dry and extruded and the red band was cut into roughly equal top, middle and bottom segments. Each was eluted with 50% ethanol and the solution concentrated under reduced pressure and made up to 10 ml. Radioactivity was measured in a solution counter and the vitamin B_{12b} concentration was estimated colorimetrically. The specific activities of the materials from the three segments were respectively 340, 360 and 360 counts/min./mg.

Partition chromatography of radioactive 'vitamin B_{12d}' and vitamin B_{12b}

The chromatogram was prepared as described above. The solute consisted of 1 mg. of radioactive 'vitamin B_{12d}' and 1·5 mg. of vitamin B_{12b}. The red band was cut into two segments; the specific activity of the material from each was 6700 counts/min./mg.

SUMMARY

1. Three red, crystalline, microbiologically and clinically active B_{12} vitamins have been isolated from *Streptomyces griseus* fermentations, namely vitamin B_{12} itself, vitamin B_{12b} and vitamin B_{12c}.

2. Vitamin B_{12c} results from the treatment of vitamin B_{12b} with nitrous acid and it contains nitrite in the molecule.

3. Removal of this nitrite group, by treatment with sulphamic acid or distillation of an acidified solution, yields vitamin B_{12b} again.

4. Absorption spectra and other physical constants have been measured.

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B_{12} Vitamins (Cobalamins)

2. NEUTRAL, BASIC AND ACIDIC COBALAMINS

By E. LESTER SMITH, S. BALL and D. M. IRELAND
Glaxo Laboratories Limited, Greenford, Middlesex

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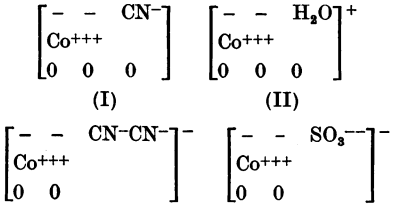
Since 1949 we have been studying the reactions of the B_{12} vitamins with sulphur dioxide, with cyanide, and with other reagents. Publication was delayed, and independent work in the same field has meanwhile been published (Fricke, Lanius, DeRose, Lapidus & Frost, 1950; Veer, Edelhausen, Wijmenga & Lens, 1950; Brink, Kuehl & Folkers, 1950; Kaczka, Wolf, Kuehl & Folkers, 1950; Beaven, Holiday, Johnson, Ellis & Petrow, 1950; Conn, Norman & Wartman, 1951; Kaczka *et al.* 1950; Kaczka, Wolf, Kuehl & Folkers, 1951).

It has become clear that the B_{12} vitamins readily form adducts with a variety of reagents in a similar manner to the haem pigments. In the light of our own and other investigations, we can now offer a more general interpretation of these phenomena than has previously been given.

It appears that there are three classes of adducts: neutral, basic and acidic. Their nature can be illustrated by the partial structures (I), (II), (III) and



(IV) (see Buhs, Newstead & Trenner, 1951). In vitamin B_{12} itself (Cyanocobalamin, I) the trivalent cobalt atom is linked to three neutral groups (0 0 0) and to three negative groups (− − CN^-), making the molecule as a whole neutral. The cyanide ion can be replaced by others with strong co-ordinating tendencies, to yield other neutral cobalamins; thus nitrite and thiocyanate give respectively nitritocobalamin (vitamin B_{12c}) and thiocyanatocobalamin. The compounds in this series are comparatively stable and can readily be obtained crystalline.

Photolysis or treatment with hydrogen and a catalyst or with sulphurous acid or certain other reducing agents, displaces the cyanide ion from vitamin B_{12} to yield vitamin B_{12b} (vitamin B_{12a} appears to be a dehydration product: see Kaczka, Wolf & Folkers, 1949; Kaczka, Denkewalter, Holland & Folkers, 1951; Cooley *et al.* 1951*a*). Vitamin B_{12b} appears to be an equilibrium mixture of hydroxocobalamin and aquocobalamin (II), the latter being the favoured form except in alkaline solution. As in vitamin B_{12b} the negative ion is replaced by the neutral molecule H_2O, the molecule as a whole acquires a positive charge, and accordingly vitamin B_{12b} titrates as a base (Kaczka, Wolf *et al.* 1950, 1951; Lester Smith *et al.* 1951; Cooley *et al.* 1951*b*).

We find that the linkage with strong acids such as sulphuric is substantially ionic in character. Accordingly, these compounds are better named as salts—aquocobalamin sulphate, etc.—rather than as sulphatocobalamin, etc. (Lester Smith, 1952).

Another member of this class of basic B_{12} vitamins is the substance called cobalichrome by Cooley *et al.* (1951*b*), in which NH_3 replaces the H_2O in aquocobalamin. The unstable adduct with elemental iodine also belongs presumably to this category.